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Synthesis of a High-Density Lipoprotein in the Developing Blue Crab (*Callinectes sapidus*)

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Abstract. An important lipoprotein in the hemolymph of crustaceans is LpI. It transports lipid to peripheral tissues and also has a role in crustacean immune recognition. We employed a monoclonal antibody specific for the LpI peptide to demonstrate by ELISA, western blot and immunohistochemistry the appearance of LpI during development of *Callinectes sapidus*, the blue crab. LpI was first found in stage 5 embryos and appeared to be synthesized by lateral basophilic cuboidal cells that demonstrated cytoplasmic immunoreactivity for LpI at their interface with the yolk mass. The embryonic cuboidal cells bore a strong cytologic resemblance to the hepatopancreas cells of later stages (zoea, megalopae, adults), which were also immunoreactive for LpI.

Introduction

The hemolymph of male and female decapod crustaceans contains a high-density lipoprotein (LpI) with concentrations ranging from 1.1 to 2.0 mg/l (Lee and Puppione, 1988; Spaziani, 1988; Lee, 1991; Spaziani and Wang, 1991; Stratakis *et al.*, 1992; Tom *et al.*, 1993; Yepiz-Plascencia *et al.*, 1995; Ruiz-Verdugo *et al.*, 1997). It plays an important role in transporting lipids from the hepatopancreas to peripheral tissues such as muscle, and functions as a β-1,3-glucan-binding protein in crustacean immune recognition (Khayat *et al.*, 1994; Hall *et al.*, 1995; Kang and Spaziani, 1995).

Embryos of *Callinectes sapidus*, the blue crab, develop in egg sacs through a series of 10 stages (Table 1) over a period of 16–23 days. At stage 10, they emerge from the egg sacs as swimming zoea larvae; these metamorphose into megalopae, then into juvenile crab forms, and ultimately become adult crabs. Until they emerge from egg sacs, embryos are nutritionally dependent on lipids and lipovitellin stored within the eggs. Lipovitellin (LpII) is a high-density lipoprotein that differs from LpI in density, sediment coefficient, and peptide components (Lee and Puppione, 1988; Lee and Walker, 1995).

In adult blue crabs, LpI is composed of phospholipids (45%), cholesterol (2%), triacylglycerols (3%), and one peptide (49%, molecular mass 112 kD) (Lee and Puppione, 1988). Although LpI was reported in juvenile and adult blue crabs, it has not been previously reported in crab oocytes or embryos. We employed a monoclonal antibody specific for the LpI peptide to demonstrate—by ELISA, western blot, and immunohistochemistry—the appearance of LpI during blue crab development. In addition, we offer immunohistochemical evidence that the developing hepatopancreas is the site of LpI synthesis in embryonic and larval stage blue crabs, and remains so in the adult.

Materials and Methods

Collection of crabs, isolation of LpI, and purification of LpI peptide

Blue crabs were collected by trawling in the estuaries near Skidaway Island, Georgia (USA). Hemolymph was collected with a 5-ml disposable syringe from the base of the swimming leg and centrifuged in a low-speed centrifuge (3 °C) for 10 min at 2000 × *g* to remove clotted materials and cells. Hemolymph lipoproteins were separated from other hemolymph proteins by adjusting the density of the

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Table 1

Description of embryo stages of Callinectes sapidus

Stage	Description	Elapsed Time (hours at 27 °C)
1	Fertilization	0
2	Early cleavage; morula (random mass of yolk cells)	12
3	Late cleavage; blastula (mass of undifferentiated yolk cells)	36
4	Embryonic naupliar stage; transparent embryo above the yolk	85
5	Early appendage formation; embryo invading ventral portion of yolk	110
6	Embryonic eye; eye appears as scarlet crescent; elongating appendages	160
7	Presence of beating heart; pigmented appendages	180
8	Oval, pigmented eye; 50% of yolk utilized; clear appendages	210
9	Compound eye with dark pigmentation; only small amounts of yolk	230
10	Protozoeae stage ready for hatching into free-swimming zoea	280

hemolymph and then centrifuging it (Beckman L5-40 ultracentrifuge, 40.3 rotor). Salt solutions used to adjust the solutions densities were prepared according to the methods outlined by Lindgren (1975). Solution densities were verified by refractometry using an Abbe refractometer (Bausch and Lomb). Consistent with an earlier study (Lee and Puppione, 1988), lipoproteins with densities less than 1.063 g/ml were not detected in blue crab hemolymph. Thus, blue crab high-density lipoprotein (Lpl) was isolated by adjusting the density of hemolymph to 1.21 g/ml with solid potassium bromide, followed by 40 h of centrifugation at 117,000 × *g*. The floating layer of high-density lipoprotein was removed and dialyzed for 24 h at 4 °C against 0.22 *M* NaCl containing 1 m*M* EDTA and 2 m*M* sodium azide. After dialysis, lipoproteins were run on vertical slab gels (7% polyacrylamide, 0.1% sodium dodecyl sulfate (SDS), 0.8% mercaptoethanol), following the procedures of Laemmli (1970). The protein band (apoLpl) was visualized with 0.3 *M* copper chloride. The apoLpl was cut from the gel and eluted by electrodialysis (Electro-Eluter model 422, Bio-Rad). The eluted apoLpl was dialyzed against 0.22 *M* NaCl. SDS- polyacryamide electrophoresis of the purified peptide was carried out to verify its purity. Purified apoLpl was used as the antigen for the preparation of monoclonal antibodies.

Monoclonal antibody production

Four female BLB/c mice were immunized with 50 μl of apoLpl (0.1 mg/ml) mixed with Freud's complete adjuvant. The injections were repeated twice (4 weeks and 6 weeks after the original injection) with 25 μg of apoLpl in Freud's incomplete adjuvant. Three days after the last injection, the mice were sacrificed and spleens removed. The mouse spleen cells were fused with a mouse myeloma strain Sp2/0, using polyethylene glycol as the fusing agent, as described by Galfre and Milstein (1981). After fusing, cells were plated in hypoxanthine/aminopterin/thymidine selection medium in microplates on a feeder layer consisting of mouse peritoneal macrophages. The wells were screened by indirect ELISA (enzyme-linked immunosorbent assay) for antibodies to apoLpl. The positive hybridomas were cloned by limiting dilution. The hybridomas were grown on RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum and 0.01% streptomycin and penicillin.

Indirect ELISA assay for apoLpl

An indirect ELISA assay was used to test antibodies, using the procedures described by Lee and Walker (1995), and absorbance was measured at 410 nm with an ELISA microplate reader (model EL307C, Bio-Tek Instruments).

Indirect competitive ELISA for apoLpl

A criss-cross serial dilution analysis was carried out to determine the optimal concentrations of apoLpl and antibody (Hornbeck *et al.*, 1992). The procedures for the indirect competitive ELISA for apoLpl using the monoclonal antibody McAb 1 are described in Lee and Walker (1995). Absorbances of the standards or extracts (A) were divided by the absorbance of the antibody not pre-incubated with antigen (A_0). The A/A_0 values for standards were plotted against the Lpl concentration on a linear-log graph to construct a standard curve. Concentrations of Lpl in egg/embryos were calculated from a standard curve prepared on the day of the assay.

Collection, extraction, and Lpl determination in oocytes

Ovaries were dissected from adult female crabs that were 12-14 d post-molt. Oocytes were washed out of minced ovaries with filtered seawater, followed by passage through nylon filters of different sizes to obtain a clean oocyte preparation. Light microscopy was used to determine oocyte diameters. After homogenization, the protein in oocyte extracts was determined by the method of Bradford (1976). Lpl concentrations in oocyte extracts were determined by competitive ELISA.

Collection, extraction, and Lpl determination in different embryo stages

Embryo stages 1 thru 10 are within an egg sac, from which the free-swimming zoea stage emerges (Table 1). The color of the sponge is indicative of the embryo stage: the color changes from bright orange to yellow to reddish

brown, and finally from dark brown to black just before hatching. Females with eggs (sponging female) at different stages were collected by trawling in the estuaries of coastal Georgia. The embryos in the sponge were staged by examination under a dissecting microscope. Using forceps, pieces of sponge were removed from a sponge-carrying female and gently shaken into 100 ml of seawater in a beaker. Embryos in a 1-ml aliquot of the water were counted to estimate the total number in the beaker, then the remaining water was filtered and the embryos collected on the filter. A hand-held homogenizer was used to homogenize 1000 embryos in 2 ml of 0.1 *M* phosphate buffer (pH 7.5). The extracts were centrifuged at 5000 × *g* for 10 min. The amount of LpI in aliquots of the supernatants (0.4 ml; 200 embryos) was determined by indirect competitive ELISA.

Collection, extraction, and LpI assay of larval forms

Zoeae were collected, extracted and LpI assays were carried out as described above. Megalopae were collected in plankton net tows in coastal Georgia estuaries during the period (spring tide in June) when megalopae enter the estuaries from offshore. They also were processed as described above.

Immunoblot (western blot) of LpI

Proteins from embryo or larval extracts were transferred from sodium dodecyl sulfate slab gels to nitrocellulose according to the methods described in Milne *et al.* (1992). After electrophoretic transfer, the membranes were probed with the monoclonal antibody (2 μg/ml) for LpI (McAbI) for 1 h, followed by rabbit anti-mouse alkaline phosphatase conjugates for 1 h. Western blue (bromochlorodindoylyl phosphate/nitroblue tetrazolium) was the chromogen (Promega). Pre-stained protein standards were used as molecular mass markers.

Immunohistochemistry

Blue crab embryos, larvae, and portions of adult hepatopancreas and ovaries were fixed in 10% neutral buffered formalin containing 1% zinc sulfate and processed for light microscopy. Standard immunohistochemical techniques (Lee and Walker, 1995) were used to probe sections of these tissues for the presence of anti-LpI immunoreactivity.

Results

Monoclonal antibody to quantify ApoLpI

Five monoclonal antibodies to apoLpI were developed. All of these antibodies reacted positively to blue crab LpI in an enzyme-linked immunosorbent assay (ELISA). One of the antibodies to apoLpI (McAbI) showed the strongest reaction to LpI. This antibody was used in an indirect, competitive ELISA to quantify LpI in oocytes, embryos, and larval forms. LpI could be detected at concentrations as low as 10 ng/ml of buffer. Using this assay and immunoblots, we found that LpI was absent from developing oocytes, newly fertilized eggs, and stages 1 through 4 embryos (Fig. 1). LpI concentrations increased steadily from stage 5 (2.3 ng/embryo, 1150 ng/ml of extract) to zoea (22.6 ng/zoea). While the concentration of LpI was increasing, yolk appeared to be decreasing such that very little yolk remained in stage 9/10 embryos. Western blots using McAbI on extracts of stages 1 through 10 showed no bands for stages 1 through, 4 and then a single immunoreactive band of increasing density (data not shown).

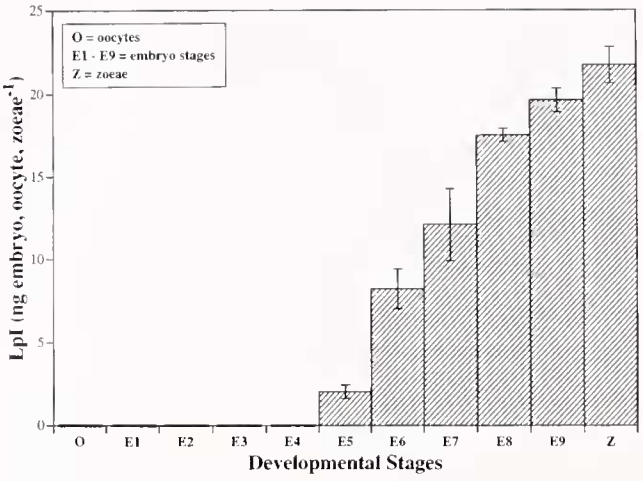

Figure 1. Amount of LpI in oocytes, different embryo stages (see Table 1 for description of each stage), and zoea. LpI determined by enzyme-linked immunosorbent assay (ELISA) as described in Materials and Methods. The values given are the mean ± standard deviation (n = 3) for 200 pooled individuals.

Immunohistochemistry using the anti-LpI antibody

Embryos. No immunoreactivity for LpI was observed in developing oocytes or in early embryo stages 1–4 (Fig. 2a). Beginning in stage 5, there were scattered immunoprecipitates within the yolk mass and at the interface with the embryonic cell mass. In addition to the morphologic changes of embryonic cell mass, individual cells dispersed throughout the yolk appeared at this time; these may represent vitellocytes. By stage 7 there were bilateral aggregates of cuboidal cells at the pole of the embryonic cell mass (Fig. 2b). The cuboidal cells had large nuclei with prominent nucleoli, moderate amounts of basophilic cytoplasm, and apical accumulations of anti-LpI immunoprecipitates. Tiny foci of anti-LpI immunoreactivity also seemed to be present in minute slit-like spaces on the non-yolk side of the cuboidal cells. By stage 10, the residual yolk was divided into bilateral masses, each encompassed by a circumference of cuboidal cells; these cells are

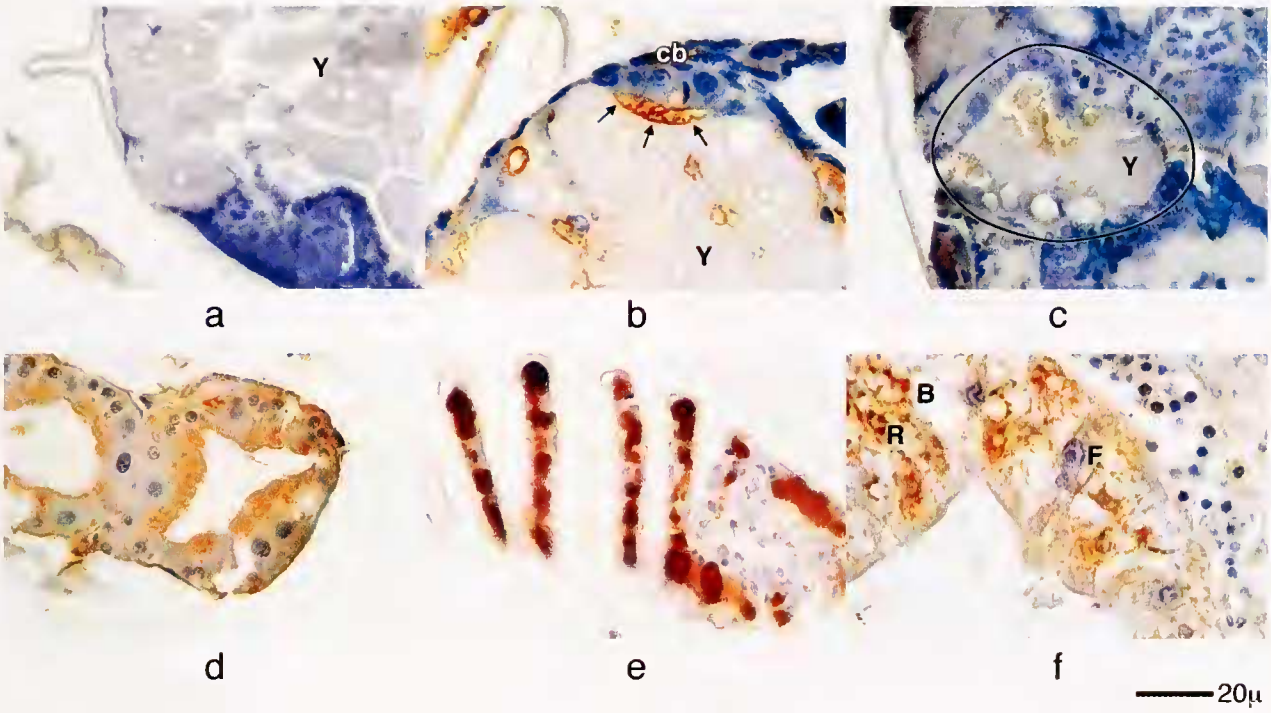

Figure 2. Immunohistochemical study using anti-Lpl Hematoxylin counterstain; original magnifications ×1000; bar = 20 μm). (a) Stage 4 embryo: Neither the embryonic cell mass nor the adjacent yolk (y) demonstrates immunoreactivity for Lpl. (b) Stage 7 embryo: Focal immunoprecipitates decorate the yolk (y) and are also localized at the interface of the lateral cuboidal cells (cb) with the yolk (arrows). (c) Stage 10 embryo (protozoea): The yolk (y) has been reduced to small bilateral masses surrounded by cuboidal cells. One such mass is delineated in this image. There are immunoprecipitates within the yolk and within the cytoplasm of the cuboidal cells. (d) Hepatopancreas of megalope: The hepatopancreas is composed of tubules of cuboidal and low columnar cells. Immunoprecipitates are concentrated at the apices. (e) Gill of megalope: The hemolymph within the vascular spaces is strongly immunoreactive for Lpl, while the background tissue elements are nonreactive. (f) Adult hepatopancreas: Immunoreactivity for Lpl is concentrated in the cytoplasm of the vacuolated (R and B) cells. In this preparation, the cytoplasm of the F cells is obscured by the more intense cytoplasmic reactivity of the R and B cells, but their nuclei (F) can still be seen.

assumed to be the anlagen of the hepatopancreas. Anti-Lpl immunoreactivity was present within the yolk residue and could also be seen in the cytoplasm of the cuboidal cells (Fig. 2c).

Larvae. The hepatopancreas of the megalopae consisted of a collection of tubular glands, less complex than the adult hepatopancreas. The glands were lined by a single layer of epithelium surrounded by a basement membrane. The lining cells were cuboidal to low columnar in shape and had basophilic cytoplasm. They resembled the anti-Lpl-reactive cuboidal cells observed in late embryo stages. The nuclei were centrally located in some cells and basally oriented in others. The nuclei of some cells had prominent nucleoli. Most cells had a cytoplasmic accumulation of granular anti-Lpl immunoprecipitate that appeared to be concentrated in the apical regions (Fig. 2d). Although occasional cells seemed to have some cytoplasmic vacuolization, they did not exhibit the definite morphologic features of cytologic specialization seen in adult hepatopancreatic cells. The hemolymph within the vascular spaces of the megalopae gills was intensely immunoreactive for Lpl, but the structural cells were nonreactive (Fig. 2e). Cellular constituents from other tissues—including muscle, eye, nerve, cuticular epithelial, and stomach—were not immunoreactive for Lpl.

Adults. The cytoplasm of lipid-rich R and vacuolated B cells in the hepatopancreas was intensely decorated with granular anti-Lpl immunoprecipitate. The non-vaculolated, basophilic F cells, in contrast, demonstrated little cytoplasmic immunoreactivity for Lpl (Fig. 2f). The F cells possessed prominent nuclei with large nucleoli, reminiscent of the nuclear features of the hepatopancreas cells of the megalopae.

Discussion

The immunohistochemical, ELISA, and western blot work support our conclusion that the lipoprotein Lpl is produced in embryonic tissues, appearing first in stage 5 embryos. In stage 5 embryos, basophilic cuboidal cells with

prominent nuclei are present at the lateral edges of the embryonic cell mass. We assume that these cuboidal cells are the site of LpI synthesis, since they demonstrate cytoplasmic immunoreactivity for LpI at their interface with yolk. Once synthesized, the LpI apparently diffuses into the adjacent yolk mass. These cuboidal cells in the embryo bear a strong cytologic resemblance to the hepatopancreas cells, which have also been shown to synthesize LpI. Lovett and Felder (1990) noted the presence of morphologically similar cuboidal cells in the larval hepatopancreas of the white shrimp, *Penaeus setiferus*.

It seems unlikely that LpI is formed directly from lipovitellin (LpII), since the two lipoproteins are biochemically quite dissimilar. They differ in molecular mass, primary amino acid sequence, and three-dimensional structure (Lee and Puppione, 1988; Yepiz-Plascencia *et al.*, 1998). Moreover, LpI does not react with antibodies to LpII (Tom *et al.*, 1993).

It has been shown in a number of arthropod groups that, during embryonic development, yolk proteins are degraded by proteases to amino acids and low-molecular-weight peptides (McGregor and Loughton, 1974; Garesse *et al.*, 1980; Ezquieta and Vallejo, 1985; Perona and Vallejo, 1985; Purcell *et al.*, 1988; Fagotto, 1990; Nordin *et al.*, 1990; Masetti *et al.*, 1998). We speculate that during embryogenesis of the blue crab, proteases and lipases in vitellophages hydrolyze lipovitellin to amino acids and fatty acids, which the cuboidal cells then use to assemble LpI.

Our evidence suggests that hepatopancreas cells of zoeae, megalopae, and adult blue crabs are involved in the synthesis of LpI, as indicated by their intense immunoreactivity. Unlike the midgut cells of the developing lobster, *Homarus americanus* (Biesiot and McDowell, 1995), the hepatopancreatic cells of the blue crab do not show light microscopic features of cytologic specialization until after the megalope stage. Thereafter, the site of synthesis may be the F-cells, since they have extensive endoplasmic reticulum and Golgi network (Al-Mohanna and Nott, 1989). After assembly in F-cells, LpI is assumed to be transported to R- or B-cells, followed by transfer into the hemolymph. These R- or B-cells may effectively concentrate LpI as suggested by their more intense immunoreactivity.

The hepatopancreas of arthropods is analogous in function to the liver (and pancreas) of vertebrates. In vertebrates, liver parenchymal cells synthesize plasma lipoproteins, with final assembly, addition of lipid, and secretion being coordinated by the Golgi apparatus (Dolphin, 1985; Havel 1987). Future planned work will determine if cuboidal embryo cells and hepatopancreas cells in other stages express apoLpI mRNA. It seems likely that R cells, which have large lipid stores, may be responsible for adding lipid to apoLpI to form LpI.

Insect and crustacean hemolymph lipoproteins differ in structure, function, and their presence or absence in the eggs. In adult insects and crustaceans, most of the hemolymph lipid is associated with high-density lipoproteins, namely lipophorin in insects and LpI in crustaceans (Gilbert and Chino, 1974; Van der Horst, 1990; Lee 1991). Lipophorin accounts for more than 50% of the total hemolymph protein in insects (Chino *et al.*, 1981), whereas LpI accounts for only 3% of the total hemolymph protein in crustaceans (Ruiz-Verdugo *et al.*, 1997).

The site of lipophorin synthesis in insects is the fat body. In females, lipophorin is transported by the hemolymph to the ovaries, where it is taken up by developing oocytes (Kawooya and Law, 1988; Kanost *et al.*, 1990). Although the major constituent of insect yolk is vitellin, a considerable amount of lipophorin is present as well. As much as one molecule of lipophorin for every 3 molecules of vitellin has been found in insect eggs (Telfer and Pan, 1988). It has been suggested that lipophorin provides a major source of lipid, and vitellin serves as a source of protein (Chino *et al.*, 1977).

Our results indicate that the site of LpI synthesis in adult and larval crabs is the hepatopancreas. There is good evidence that, like lipophorin in insects, LpI is an important carrier of lipid to the ovary in the adult (Harry *et al.*, 1979; Telfer *et al.*, 1991; Ravid *et al.*, 1999). However, whereas LpI may transfer its lipid to the ovary and then recirculate, as apoLpI, to the hepatopancreas to pick up more lipid, in insects, the lipophorin, after losing some of its lipid, is transferred into the developing oocytes (Harry *et al.*, 1979; Telfer *et al.*, 1991).

There are other important differences between insect lipophorin and crab LpI. Lipophorin has a high diacylglycerol content, while phosphatidyl choline is the major LpI lipid. There are also important differences in the major yolk protein, vitellin, of insects compared with the major yolk protein, lipovitellin (LpII), of crabs. Insect vitellin is 7%–10% lipid, with the lipids being primarily diacylglycerol and phospholipid (Beenakkers *et al.*, 1985; Wheeler and Kawooya, 1990). Crab lipovitellin is 40%–50% lipid, most of which is phospholipid (Lee and Puppione, 1988; Lee and Walker, 1995).

Differences in function may explain the presence of lipophorin in insect embryos and the absence of LpI in early stages of developing crustaceans. As noted above, both lipophorin and LpI apparently carry lipid to the ovary. However, LpI is also involved in the adult crustacean immune system, whereas lipophorin is not known to have such a role in insects. We hypothesize that the importance of LpI in late crab embryo stages and in larvae is related to its function in the developing immune system. The role of LpI in lipid transport may not become critical until the juvenile stages.

Acknowledgments

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